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14. ABSTRACT

In this project, we focus on oxidation and glycosylation PTMs on proteins known to be secreted by the breast as candidate biomarkers for the early detection of breast cancer. ELISA microarray technology is employed to evaluate assays that have potential be used as breast cancer biomarkers. During the second year of this project we have continued the validation for our PTM-ELISA microarray. Overall, our data suggest that the PTM ELISA microarray platform is a promising tool for discovery and evaluation of biomarkers that have potential for the early detection of breast cancer. Besides the post-translational modification, we also investigated post-transcription factors (microRNAs) for breast cancer studies. Our studies suggested that microRNAs may be a potential promising biomarkers for breast cancer.

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ELISA, microarray, Post-translationallymodifications(PTMs)

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Introduction

Breast cancer is the fifth most common cause of cancer death. Surgery removal of breast cancer (mastectomy or lumpectomy), has dramatically reduced breast cancer mortality. However, more accurate screening method that can detect breast cancer in earliest stages is essential for these surgeries. Early breast cancer detection is to find cancers before they start to cause symptoms. Mammography is the current standard test for breast cancer screening although many false positive results have been reported (*1, 2*). Magnetic resonance imaging (MRI) is also recommended along with mammograms for some women at high risk for breast cancer. Ultrasound and other tests may also be helpful for some women. Current screening methods, including clinical breast examination and conventional mammography, have high rates of false-positive and false negative results (*3-6*). Positron Emission Tomography (PET**)** is a newly developed imaging exam of the breast. It uses an FDA-approved sugar attached to a radioactive particle to detect cancer cells. PET may be able to detect breast cancer before it can be seen with mammograms and may be as good as or better than breast MRI.

Conventional enzyme-linked immunosorbent assay (ELISA) methods are widely used to screen many potential diseases based on changes in blood proteins. Changes in proteins identified by proteomic studies are largely different from those found by genomics studies (*7, 8*). This underscores the importance of performing biomarker screens at the protein level and

Table1 Subjects Information from Group 1

	Healthy Controls	Benign	Invasive Breast Cancer	Total
Subjects (N)	22	22	24	68
All ♀				
Age (Average ±SEM)	46±16	52±14	50±8	50±13
White	15	16	21	53
Race				
No white	7	6	3	16
BMI (kg/m^2)	23 ± 1 (2)[a]	26 ± 3 (5)[a]	26 ± 4 (12)[a]	26 ± 4 (19)[a]

[a] Only limited subjects (number listed in brackets) have available BMI information.

Table 2, Subjects Information from Group 2

	Benign	Invasive Breast Cancer	Total
Subjects (N)	54	38	92
Age (Average ±SEM)	53±13	53±11	53±12
White	54	38	92
Race			
Non-white	0	0	0
BMI (kg/m^2)	26 ± 5 (24)[a]	30 ± 8 (25)[a]	28 ± 7 (49)[a]

[a] Only limited subjects (number listed in brackets) have available BMI information.

suggests that many of the differences between normal and cancer samples are due to posttranslational modifications (PTMs) such as phosphorylation, glycosylation, oxidation, methylation, ubiquitinylation and acetylation (*9*). ELISA microarray technology is employed to evaluate assays that have potential be used as breast cancer biomarkers (*10-15*). During the first year of this project we have validated a panel of ELISA capture antibodies for our PTM-ELISA microarray. And we tested 8 proposed PTM detection antibodies with clinical breast cancer plasma samples. During the second year of this project, we analyzed more clinical plasma samples, and narrowed down several potent PTM biomarkers (GSH, Globo-H, 4-HNE) in

ELISA microarry for breast cancer early detection. We also investigated potent new biomarkers (microRNAs) in breast cancer cell lines studies, compile these new biomarkers along PTMs for breast cancer early detection. And we discussed future PET applications based on these new discovered biomarkers (including PTMs and microRNAs).

Body

Develop and validate PTM-ELISA microarray The microarray sandwich ELISA is exceptionally sensitive for clinical screening (*14*). The microarray technique is also powerful for targeted discovery research because of its ability to simultaneously conduct multiple assays. At the same time, this multiplex analysis requires much less sample (15-20 μL per chip, after a 5-fold dilution) than conventional ELISA, thereby allowing the screening of many PTMs using very small sample volumes. The new ELISA microarray has developed for PTM analysis based

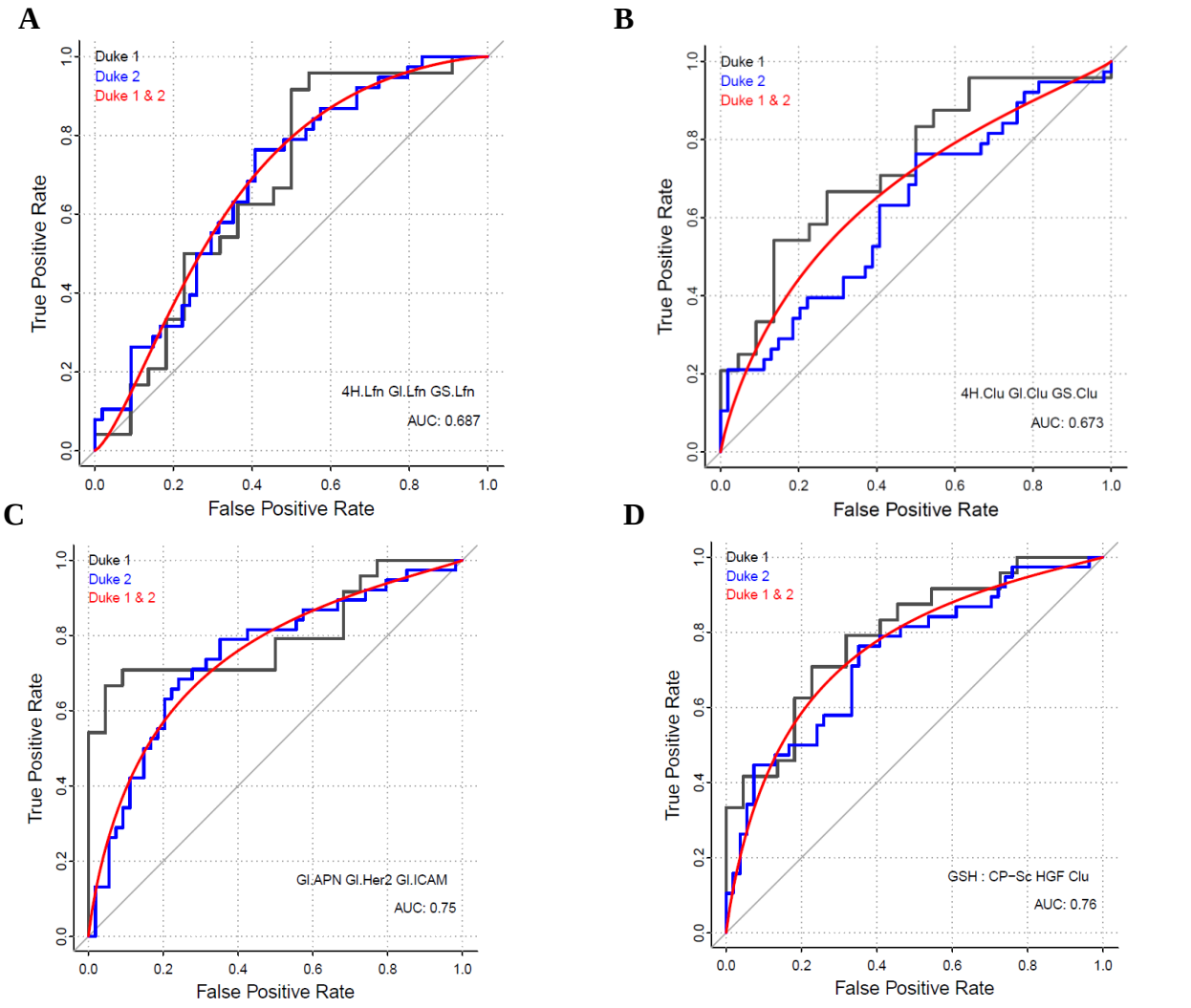

Figure 1 ROC (receiver operating characteristic) curve analysis for PTM-ELISA microarray results from two independent sets of early breast cancer plasma samples (Duke1 & Duke2). Combination of three different PTMs biomarkers: 4HNE (4H),Globo-H (Gl) and GSH (Gs) within same capture antibody Lactoferrin (Lfn) in **panel A**, or Clusterin (Clu) in **panel B** give AUC (area under the curve) 68.7% and 67.3% respectively to distinguish invasive breast cancer from the healthy controls. Combination of three different capture antibody: Aminopep-tidase N (APN)), Her2 and ICAM within same PTM: Globo-H (Gl) gives AUC 75% **(panel C).** Combination of three different capture antibodies: Ceroplasmin (CP-Sc), Human Growth Factor (HGF) and Clusterin (Clu) within same PTM: GSH (Gs) gives AUC 76% **(panel D)**.

on the regular ELISA microarray platform, with the major difference being the detection antibody. That is, we use biotinylated antibodies that specifically recognize the PTMs. For this purpose, we adapted the established capture antibodies from the panel and added more capture antibodies based on previous studies for those abundant antigens that only express in breast cancer plasma but not from healthy controls (*15*). We validated these capture antibodies by individual ELISA microarray and by multiplex combination. We tested them together for optimizing the specificity and sensitivity followed the standard protocol. We validated our PTM-ELISA microarry with two groups of human plasma samples collected from Duke University (Table 1). All subjects were recruited and samples were collected under IRB-approved protocols and informed consent at the Duke University. These protocols were reviewed by the Institutional Review Board of the Pacific Northwest National Laboratory prior to transfer and analysis of the samples. Two groups of plasma samples, for a total of 160 subjects, were analyzed. First group contains plasma from healthy controls, benign tumor, and invasive breast cancer (total n = 68). Second group contains plasma from benign tumor, and invasive breast cancer (total n = 92). Samples from early stage breast and controls were matched based on the subject's age, body mass index (BMI) and race (Table 1). The samples were also blocked based on the study group to ensure that the spatial distribution of the samples on the chips and slides would not bias the study results.

As we stated in our proposal, at the first year of this project, we have evaluated oxidation modifications using the new PTM microarray chip. For oxidative modifications, 4-hydroxynonenal (4HNE), nitrotyrosine, and glutathione (GSH) adducts were tested with commercial available antibodies and antigens to select the best specificity and sensitivities. And the small number of breast cancer plasmas samples was applied to establish the assays. We also developed halotyrosine antibodies for the breast cancer early detection.

Previously, the lab from Dr. Zangar in PNNL has developed an ELISA microarray chip with a set of 20 assays for proteins known to be altered in blood from breast cancer patients (*10, 13, 16*). BSA, PBS, and IgG fragment also are printed as negative controls. Green fluorescent protein (GFP) is spiked into each sample and used as an internal calibrant. Processing the breast cancer samples for 5 different PTMs and 27 different antigens has produced a total of 135 assays.

Our preliminary studies indicated that there is a correlation between the breast cancer and the level of nitrotyrosine tested with human plasma samples. And we expanded this assay for several other similar studies for tyrosine modifications. Because tyrosines that are post-translationally modified by nitrating oxidants, brominating oxidants, and chloramination oxidants produced from macrophages, neutrophils or eosinophils to form nitrotyrosine, bromotyrosine and chlorotyrosine respectively, have relevance to breast cancers (*17-23*). For example, hypochlorous acid/hypochlorite adducts have been reported for breast cancer (*24-27*). Our studies suggest that circulating PTM levels can be used as a biomarker for endothelial cell dysfunction, which is of concern in several human diseases. We have published nitrotyrosine plasma samples studies to the journal of ***Environmental Health Perspectives*** (Appendix page 13).

4HNE is a non-enzymatic breakdown product of lipid peroxides (*28*). These modifications have been reported to be associated with oxidative enzymatic breakdown product of lipid peroxides (*28*). These modifications could be associated with oxidative stress associated with the immune response (*21, 28*). When we established this PTM-ELISA microarray assay, and tested with breast cancer plasma samples, we found 4HNE protein modification is alerted early breast cancer with several circulating proteins (i.e. PDGF, HGF) (Figure 1, Appendix, page 13).

Glutathione (GSH) is protein adduct which is an indicative of intracellular oxidative stress, especially in the endoplasmic reticulum. Growing evidence suggested that GSH adducts is useful indictor of breast cancer (*29-31*). We established this PTM-ELISA microarray chip, and tested it

with clinical breast cancer plasma samples, we found GSH modification is altered with several circulating proteins (Figure 1, Appendix, page 13).

We developed GloboH-ELISA microarray assays, and tested it with clinical breast cancer samples. We found several circulating proteins containing this modification are altered in invasive cancer (Appendix, page 13). We are now in the preparation of the manuscript of this study. For the other glycosylation modification PTM-ELISA-microarrays, we are lacking of specific antibodies currently, hopefully we can find applicable antibodies for those glycosyations in future. Based on the development of these methods, we published one method paper on ***Current Protocol in Toxicology***. (Appendix, page 18).

microRNA and cancer biomarker Recently, there is growing body of evidence, in addition to alterations in protein-encoding genes, or protein post-translational modifications as biomarkers for breast cancer, abnormalities in non-coding RNAs are largely reported for breast cancer biomarkers (*32-39*). In particular, a class of small cellular RNAs, termed microRNAs (miRNAs), acting as agents of the RNA interference pathway, can lead to silencing of their cognate target genes, doing so either by cleaving mRNA molecules or by inhibiting their transcription or translation (*40, 41*). We investigated microRNA molecules for breast cancer biomarkers by measuring the PTEN pseudogenes transcription level. This assay was adapted from Professor Pandolfi at Harvard Medical School. The microRNAs (mir-17, mir-19, mir-21, mir-26 and mir-214) target the PTEN pseudogene UTR regions and thus regulate the PTEN and PTEN pseudogene transcription. The pGlu luciferase plasmid expressing the wild type PTENP1 3'UTR (pGLU/ψ3'UTR) or the 3'UTR in which the seed matches of the 5 PTEN-targeting microRNAs have been mutagenized (pGLU/ψ3'UTRmut). The interactions between microRNAs to the PTEN pseudogenes can be investigated by simply transfection of these two plasmids into breast cancer cell lines. We tested breast cancer cell line MCF-7 and human mammary epithelial cell MCF-10A (Figure 2). And we found that the transcription level of the PTEN pseudogene is significantly increased in breast cancer cells compared to epithelial cells (Figure 3). This result suggested that these micrRNAs are largely involved in the post-transcription regulation of PTEN pseudogene. And the microRNAs (mir-17, mir-19, mir-21, mir-26 and mir-214) may be useful biomarkers for differentiate the breast cancer cells from regular human epithelial cells.

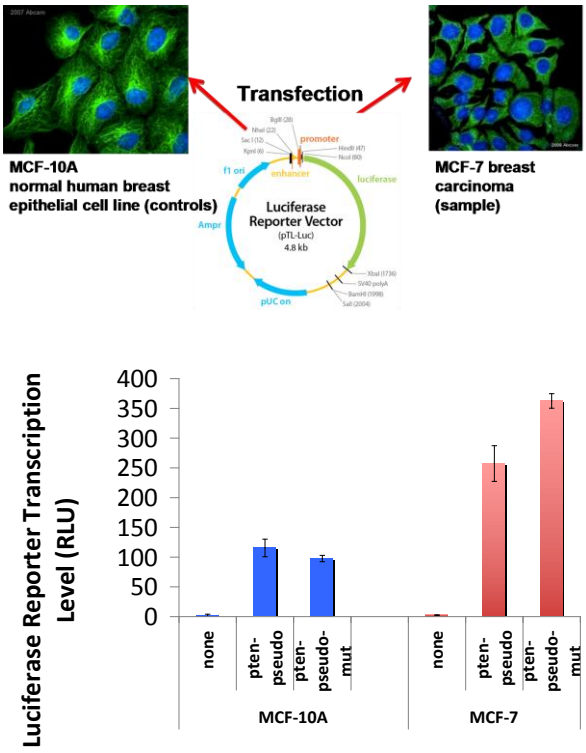

Figure 2 microRNAs for breast cancer biomarker Luciferase report plasmid for assay of PTEN pseudogen transcription level (top). Different transcription level of PTEN pseudogene were found between breast cancer cell (MCF-7) and epithelial cell (MCF-10A).

Regarding the microRNA uptaking by breast cancer cell lines, we investigated the other important microRNA-10b in the established breast cancer metastasis model. A recent screening of microRNAs towards inhibition of breast cancer metastasis ended with several anti-micoRNA

reagents in vitro cultivated cells and animal models (*40, 42, 43*). For example micro-RNA-10b (mir-10b, located on Hox gene clusters, which are critical for the proper number and placement of embryonic segment structures.) were identified for its role in positively regulates breast cancer cell migration and invasion by the transcription factor Twist (*42*). HOXD10 was reported to be a direct and functional target of mir-10b (*40, 42, 44*). RNA molecules of anti-mir-10b were found 10-fold reduction of invasive properties of MDA-MB-231 breast cancer cells (*42*). These studies established that anti-mir-10b RNA can be directly used to inhibit the breast cancer metastasis. However those small RNA molecules cannot be directly uptaken by cells, several transfection techniques are applied to ensure successful intaking of these anti-mir RNA reagents into cultured cells or animal tissues (*45-47*). For example cationic liposomes are widely used for DNA vectors and RNAi delivery, but with low efficiency of transfections (*48*). Alternatively synthetic polymers and nano particles are applied in gene delivery in recent years because of their potential advantages of low immunogenicity (*49, 50*). Poly (L-Lysine) (PLL) has been widely used as a gene delivery carrier because its excellent nuclei acid condensation property and efficient protection of nuclei acid from the attack of nucleases(*49, 51-53*). We used synthesized PLL (MW 40,000) to deliver anti-mir-RNA-10b molecules on the breast cancer cell MDA-MB231. We found that unmodified RNA molecules have very strong interactions with PLL and the formed nanoparticles can deliver the RNA into breast cancer cytoplasm (Figure 3).

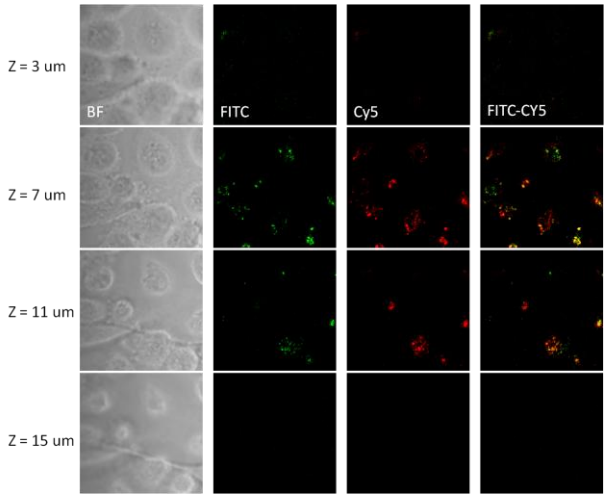

Figure 3. 50 nM PLL-anti-mir-10b complex were added into MDA-MB-231 cells. After 28 hours, the cell monolayers were washed with 1XPBS three times. Images were taken by Zeiss 710 laser scanning confocal microscope. FITC-labeled PLL (green), Cy5-labeled anti-mir-10b (red), and FITC-Cy5 merged view were visualized at 3 µm, 7 µm, 11 µm, and 15 µm Z-dimensions.

Progress according to our Specific Aims and Statement of Work

Specific Aim 1) Develop antibody microarray chips that can be used to measure PTMs associated with glycosylation (GloboH, TF Antigens, AGEs) state or oxidative adducts (nitrotyrosine, 4-HNE, GSH). For this specific aim, we have successfully developed 5 PTM assays based on selected biomarker panels. These chips are targeting abundant proteins that have been identified in NAF and are also found in the circulation. The 5 PTM detection antibodies have been individually used with the full panel of 24 capture antibodies to detect specific PTMs on each of the antigens.

Specific Aim 2) Evaluate the utility of the panel of biomarkers developed in Aim 1 in a case-control study with plasma samples. We have tested our PTM-ELISA microarray chips with prelinamry one set of 68 clinical plasma samples containing early breast cancer and benign controls. We reported thoses prelinary findings in last annual report.

Specific Aim 3) Independently validate the best combination of assays identified in Aim #2 by evaluating two additional sets of plasma samples. The sensitivity and the specificity of each assay and the combined assays will be evaluated and compared to the results in Aim #2Investigated other possible potent breast cancer biomarkers. Up to this year, totally 150

samples from two sets of human plasma samples were analyzed. Each sample was tested in triplicate. So we have tested totally (24X5X150X3) 54,000 sandwich ELISA for this project.

Statement of Work Task 1. "Develop antibody microarray chips that can be used to measure PTMs associated with glycosylation (GloboH, TF Antigens, AGEs) state or Oxidation adducts (nTyr, 4-HNE, GSH)". We have successfully developed microarray chips for GloboH, 4-HNE, GSH and nitrotyrosine. And we also developed halotyrosine antibody and halotyrosine microarray chips. Although we have not found any significant changes with nitrotyrosine or halotyrosine with tested breast cancer plasma samples, we may apply these chips with breast cancer tissue or other types of samples in future.

Statement of Work Task 2. "Optimize the combination panel of selected biomarkers from the Aim 1 using serum samples with and without breast cancer". The initial panel of 27 biomarkers may be reduced to a small group of the most sensitive and specific assays. We have finished initial test of each PTM assay using 68 plasma samples. And we refined the combination of assays by eliminating the capture antibodies that did not distinguish invasive breast cancer from normal women.

Statement of Work Task 3. "Independently validate the biomarker panel developed in Aims 1 and 2 by evaluating two additional sets of human plasma samples". We just finished two sets of samples analysis with the developed PTM-ELISA microarray. These samples were from women with early stage breast cancer. Control samples were from women that are matched based on age and age, menopausal status and body mass index. The sensitivity and the specificity of each set of PTM assays were evaluated individually and in combination with the analyses using other PTMs. The best combination results were presented in Figure 1.

Statement of Work Task 4. "Investigate the fundamental biochemistry of the most promising breast cancer biomarkers". As we stated in our original statement of work 4, we further analyzed PTMs within the positive "outcomes". The immunoprecipitation (IP) and western blotting were applied to verify. However, with the current antibodies for Globo H, and nitrotyrosine, the IP protocol has not worked out yet. To elucidate the molecular mechanism of the early breast cancer, other potential biomarkers, such as the microRNAs, were investigated. Our preliminary results suggested that a group of microRNA targeting of PTEN pseudogene are promising indicator to differ breast cancer cells (MCF-7) from human epithelial cells (MCF-10A).

Difficulties

Identifying protein biomarkers in serum or plasma remains a big challenge given the vast range of protein species and broad range concentrations. We intend to develop microarray chips to find whether oxidation and glycosylation modifications can be used for breast cancer detection biomarkers. The big difficulty for this type chip development is hard to find applicable antibodies for the PTM-ELISA. We developed a halotyrosine monoclonal antibody for the assay development, but we have not identified any plasma protein modification differ between breast cancer and benign controls using this antibody. And the immunoprecipitation protocol has not worked out due to lacking of more specific antibodies for those PTMs.

Discussion for new tracer development for breast cancer early detection

Through the high through-put ELISA microarray assay, we identified several important PTMs biomarkers for breast cancer early detection. But the application is limited by the specific antibodies. As the PI of this project has moved to Washington University at St. Louis under the supervision of Dr. Zhude Tu, who has led many years of radiotracer development for breast cancer studies using the PET. This provides the opportunities of developing a specific radiotracer

that is able to recognizing these PTM modifications (4-HNE, GSH, Globo-H). If successful, our finding will greatly benefit the breast cancer early detection. And at the meantime, our preliminary microRNA studies suggested microRNA may also be applied for post-transcription biomarkers for breast cancer. We will also test if any of these micrRNA molecules can be used as molecular for radiolabeling with suitable isotopes for early detection of breast cancer.

Key Research Accomplishments

1. Validated panel of 24 ELISA analysis for breast cancer studies;
2. Validated PTM-ELISA microarray chips for nitrotyrosine, 4HNE, GSH, and GloboH
3. Validated GSH, 4HNE, halotyrosine, and PTM-ELISA microarray chip;
4. Retested the PTM-ELISA microarray chips on two sets of clinical plasma samples;
5. micrRNA-PTEN pseudogene
6. microRNA-10b PLL breast cancer uptaking

Reportable Outcomes

Published Peer Reviewed papers

- Jin, Hongjun, Y. Yu, W.B. Chrisler, Y. Xiong, D. Hu, and C. Lei, Sustainable inhibition of breast cancer metastasis by delivery microRNA-10b to breast cancer cell line using polylysine nanoparticles, ***Breast Cancer: Basic and Clinical Research*** 2011 6:1-6

- Jin, Hongjun, B.J. Web-Robertson, E.S. Peterson, R. Tan, D.J. Bigelow, M.B. Scholand, J.R. Hoid, J.G. Pounds, and R.C. Zangar Smoking and COPD have Opposite Effects on 3-Nitrotyrosine in Plasma Proteins ***Environmental Health Perspectives***, 2011, 119:1314–1320.

- Jin, Hongjun and Richard C. Zangar Invited Review: "Multiplex ELISA Microarray protocol for 3-Nitrotyrosine Detection", **John Wiley & Sons, Inc, Current Protocols in Toxicology**, 2012 Unit Number: Unit 17.15, DOI: 10.1002/0471140856.tx1715s51,Online Posting Date: February, 2012

Manuscript submitted:

Jin, H, T.S. Hallstrand**,** D.S. Daly, P. Nair, D.J. Bigelow, J. G. Pounds and R.C. Zangar Halotyrosine levels in induced sputum reflect eosinophil activity and indirect airway hyperresponsiveness ***American Journal of Respiratory and Critical Care Medicine***

Posters

AACR 2011 Abstract #3182 Jin H., DS. Daly, R. Tan, J.R. Marks and R.C. Zangar, poster presentation, "PTM ELISA Microarray for Breast Cancer Biomarker Discovery", Abstract #3182, American Association of Cancer Research 102 Annual Meeting,April 2-6, 2011, at the Orange County Convention Center in Orlando, Florida (Appendix, page 11)

Era of Hope 2011, Abstract, Posttranslational Modifications of Specific Circulating Proteins are Promising Biomarkers for Breast Cancer Detection (Appendix, page 12)

American Thoracic Society International Conference 2011
[C35] INSIGHTS INTO LUNG DISEASE USING NOVEL METHODS
Halotyrosine Modifications In Sputum Proteins Are Indicative Of Asthma Severity
Poster - F137 Location Area F, Hall B (Upper Level), Colorado Convention Center
Categories 07.02 - Eosinophils Neutrophils (AII), Poster - F137

Conclusion

To detect breast cancer in its earlier stages, oxidantion and glycosylation modification PTMs are proposed as potential biomarkers with circulating human proteins. We developed 5 PTM-ELISA microarray chips (nitrotyrosine, halotyrosine, 4HNE, GSH and GloboH) compiled with an established panel of 24 capture antibodies compiled with previous breast cancer studies. Using these approaches, we identified several circulating human proteins-containing PTMs unique to the plasma of breast cancer patients. We validated the PTM-ELISA microarray with two independent sets of total 150 human plasma. And ROC analysis suggested that best combination PTMs of Globo H, GSH and 4-HNE can reach AUC to 76% from this study. This work provides a methodological platform for the study of post-translationally-modified proteins. Through this novel PTM-ELISA microarray screening, we found that Globo H, 4HNE and GSH may be potential useful biomarkers for breast cancer early detection.

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Appendices

Conference Abstracts

AACR 2011

Abstract #3182 (Accepted)
PTM ELISA microarray for breast cancer biomarker discovery
AACR 102 Annual Meeting
(April 2-6, 2011, at the Orange County Convention Center in Orlando, Florida)

PTM ELISA Microarray for Breast Cancer Biomarker Discovery

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Post-translational modifications (PTMs) of proteins are known to be altered during breast cancer development. These PTMs are potentially useful biomarkers for breast cancer. In order to study the potential of PTMs in the early detection of breast cancer, ELISA microarray protocols for analyzing several PTMs in a panel of 24 proteins that are found in blood but known to be secreted by breast tissue and/or breast tumors. We evaluated these PTMs in a case-control study with 68 plasma samples. The control samples were from age-matched women with benign breast disease. The sensitivity and the specificity of each assay and the combined assays were evaluated using the areas under (AUC) the receiver operator curves. In the best multivariate analysis, the AUC values ranged from 77% to 87% for 4 different PTMs. Overall, our data suggest that the PTM ELISA microarray platform is a promising tool for discovery and evaluation of biomarkers that have potential for the early detection of breast cancer. (* This research is supported by NIH U01 CA117378 and The US Department of Defense BCRP Postdoctoral Fellowship (W81XWH-10-1-0031).

Era of Hope 2011

Abstract (Accepted)

Posttranslational Modifications of Specific Circulating Proteins are Promising Biomarkers for Breast Cancer Detection*

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Levels of post-translational protein modifications (PTMs) are known to be altered in breast cancer tissue. We therefore hypothesized that PTMs in proteins that are secreted by breast tissue could be useful circulating biomarkers for breast cancer. In order to study the potential of specific PTMs for the early detection of breast cancer, an ELISA microarray platform for the analysis of PTMs was developed. This platform included an ELISA microarray chip for 24 proteins that are detectable in blood but are secreted by breast tissue and/or breast tumors. For each of these 24 proteins, we measured the levels of four candidate PTMs in two independent sample sets with a total of 140 plasma samples. For both sample sets, all samples were collected at the time of biopsy, after referral from a positive screen (such as mammography). The samples were subsequently categorized as either benign controls or cancer cases based on the pathology report. The subjects in the two groups were age-matched. The sensitivity and the specificity of each assay and the combined assays were evaluated using the areas under the receiver operator curves (AUC). In an analysis of 4 markers, the best AUC value was 87%. Our study suggests that certain circulating proteins with specific oxidative modifications or glycosylation residues can be used to distinguish between women with early breast cancer and those with benign breast disease. Therefore, these biomarkers may have potential to distinguish between true positive and false positive results obtained with standard clinical screens, including mammography. (* This research is supported by NIH U01 CA117378 and The US Department of Defense BCRP Postdoctoral Fellowship (W81XWH-10-1-0031).

American Thoracic Society International Conference 2011
[C35] INSIGHTS INTO LUNG DISEASE USING NOVEL METHODS
Halotyrosine Modifications In Sputum Proteins Are Indicative Of Asthma Severity
Poster - F137
Location Area F, Hall B (Upper Level), Colorado Convention Center
Categories 07.02 - Eosinophils Neutrophils (AII), Poster - F137

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Description

RATIONALE: Eosinophils are believed to contribute to asthma development and exacerbations. When activated, eosinophils release eosinophil peroxidase, which produces hypobromite (HOBr). Hypobromite reacts with proteins to form bromotyrosine residues. Studies on total bromotyrosine levels have shown that these protein modifications are increased in asthmatics, but are decreased in response to anti-inflammatory drugs. Studies on bromotyrosine have been hampered by the lack of an antibody that recognizes 3-bromotyrosine, the predominate bromotyrosine modification in vivo. METHODS: Using a novel protocol for antigen generation and hybridoma selection, we generated a monoclonal antibody that recognizes 3-bromotyrosine, dibromotyrosine, and related chlorotyrosine analogs. Since this antibody apparently reacts with all naturally occurring, halogenated tyrosine protein modifications, we call this a halotyrosine antibody. The halotyrosine antibody was used as the detection reagent in a sandwich ELISA microarray platform. This platform was used to measure the halotyrosine levels in 24 proteins in 15 sputum samples that were equally divided (5 samples per group) between healthy controls or asthmatics with either high or low sputum eosinophils counts. RESULTS: Data indicated that halotyrosine levels of four proteins were significantly increased in the sputum samples from both of the asthma groups compared to the controls. There was not, however, any significant difference between asthmatics with high and low eosinophil counts. Interestingly, the halotyrosine levels correlated (r2 ~ 0.5) with exercise-induced bronchoconstriction measures of asthma, but the eosinophil counts did not. CONCLUSIONS: We have developed a novel antibody that can be used for measuring halotyrosine levels in sputum proteins. Preliminary analyses indicated that halotyrosine levels in individual sputum proteins may be a useful indicator of asthma severity. (This research was supported by the National Institute of Environmental Sciences (U54/ES016015) and a US Department of Defense postdoctoral fellowship (W81XWH-10-1-0031; HJ), and NHLBI R01HL089215 (TSH). Dr Nair is supported by a Canada Research Chair in Airway Inflammometry. The mepolizumab study was supported by an unrestricted grant-in-aid from GlaxoSmithKline Canada.)

Published Peer Reviewed paper in
Environmental Health Perspectives

Research

Smoking, COPD, and 3-Nitrotyrosine Levels of Plasma Proteins

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Background: Nitric oxide is a physiological regulator of endothelial function and hemodynamics. Oxidized products of nitric oxide can form nitrotyrosine, which is a marker of nitrative stress. Cigarette smoking decreases exhaled nitric oxide, and the underlying mechanism may be important in the cardiovascular toxicity of smoking. Even so, it is unclear if this effect results from decreased nitric oxide production or increased oxidative degradation of nitric oxide to reactive nitrating species. These two processes would be expected to have opposite effects on nitrotyrosine levels, a marker of nitrative stress.

Objective: In this study, we evaluated associations of cigarette smoking and chronic obstructive pulmonary disease (COPD) with nitrotyrosine modifications of specific plasma proteins to gain insight into the processes regulating nitrotyrosine formation.

Methods: A custom antibody microarray platform was developed to analyze the levels of 3-nitrotyrosine modifications on 24 proteins in plasma. In a cross-sectional study, plasma samples from 458 individuals were analyzed.

Results: Average nitrotyrosine levels in plasma proteins were consistently lower in smokers and former smokers than in never smokers but increased in smokers with COPD compared with smokers who had normal lung-function tests.

Conclusions: Smoking is associated with a broad decrease in 3-nitrotyrosine levels of plasma proteins, consistent with an inhibitory effect of cigarette smoke on endothelial nitric oxide production. In contrast, we observed higher nitrotyrosine levels in smokers with COPD than in smokers without COPD. This finding is consistent with increased nitration associated with inflammatory processes. This study provides insight into a mechanism through which smoking could induce endothelial dysfunction and increase the risk of cardiovascular disease.

Key words: cigarette smoke, COPD, ELISA, eNOS, nitrotyrosine, posttranslational modification. *Environ Health Perspect* 119:1314–1320 (2011). http://dx.doi.org/10.1289/ehp.1103745 [Online 6 June 2011]

Cigarette smoking is a major risk factor for cardiovascular and lung diseases such as chronic obstructive pulmonary disease (COPD) and cancer (Iribarren et al. 1999). Similarly, environmental or secondhand exposure to cigarette smoke is a major health concern. For example, in the United States in 2005, it was estimated that environmental smoke exposure caused 3,000 deaths from lung cancer and 46,000 deaths from coronary artery disease (U.S. Department of Health and Human Services 2006), suggesting that cardiovascular disease is the greatest source of smoke-related mortality for low-dose exposure. Increased inflammatory stress and endothelial dysfunction are believed to be the primary mechanisms of smoking-related cardiovascular disease (Munzel et al. 2006).

Endothelial function is largely regulated by nitric oxide, a gas that acts as a key physiological regulator of blood pressure. In endothelial cells, a single nitric oxide synthase (eNOS) is responsible for nitric oxide production (Kubes et al. 1991). This nitric oxide rapidly diffuses into the vascular smooth muscle cells, where it indirectly stimulates relaxation of blood vessels through regulation of contractile proteins via activation of guanylate cyclase (Beckman and Koppenol 1996) or of sarcoplasmic/endoplasmic reticulum calcium ATPase (Adachi et al. 2004). The relaxation of the smooth muscle results in a decrease in vascular resistance and blood pressure and a concomitant increase in blood flow (Loeb and Longnecker 1992; McPherson et al. 1995).

Although nitric oxide is a free radical, it is not a highly reactive one (Beckman and Koppenol 1996; Church and Pryor 1985). An important route of nitric oxide degradation is the rapid reaction with superoxide anion to form the more reactive product, peroxynitrite ($ONOO^-$). Peroxynitrite reacts with proteins to form 3-nitrotyrosine (Beckman and Koppenol 1996). Immune cells, including macrophages and neutrophils, simultaneously release nitric oxide and superoxide into phagocytic vacuoles as a means of generating peroxynitrite to kill endocytosed bacteria (Pacher et al. 2007). Other inflammatory cells can also produce reactive chemicals that can result in nitrotyrosine formation, including the peroxidases in activated neutrophils and eosinophils. Thus, protein-associated nitrotyrosine is a characteristic marker of nitrative stress and, commonly, inflammation (Pacher et al. 2007). For example, increased levels of protein-bound nitrotyrosine have been reported in a variety of human diseases with an inflammatory component, including atherosclerosis, myocardial ischemia, multiple sclerosis, Parkinson's disease, inflammatory bowel disease, amyotrophic lateral sclerosis, diabetes, and many others, as reviewed by Pacher et al. (2007).

Cigarette smoking alters the concentration of exhaled nitric oxide in a time-dependent manner. One minute after smoking cessation, levels of exhaled nitric oxide are increased in smokers (Chambers et al. 1998). Exhaled nitric oxide levels remain statistically elevated at 10 min after smoking, but these values are closer to control levels than to the values obtained at the 1-min time point (Chambers et al. 1998). This transient increase in exhaled nitric oxide probably reflects the fact that cigarette smoke contains up to 300 ppm nitric oxide (Pryor and Stone 1993). Thus, there is a rapid uptake of nitric oxide by the lung during cigarette smoke exposure, followed by a rapid loss after smoking. Two hours after smoking, however, exhaled nitric oxide levels are reduced in smokers relative to controls (Kanazawa et al. 1996). Thus, it seems evident that smoking has a sustained suppressive effect on exhaled nitric oxide levels that is not directly dependent on nitric oxide uptake from cigarette smoke. This sustained reduction in exhaled nitric oxide in smokers may result from inflammatory processes and superoxide-dependent conversion to peroxynitrite (Peluffo et al. 2009). In this conceptual model, the increase in peroxynitrite is predicted to elevate nitrotyrosine levels. One small study (n = 5 for the smoking group) found a statistically significant increase in nitrotyrosine levels in blood proteins from smokers compared with nonsmokers, thus supporting the superoxide-degradation model (Peluffo et al. 2009). Studies in animals have also detected an increase in nitrotyrosine in circulating proteins after exposure to cigarette smoke (Kunitomo

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The authors declare they have no actual or potential competing financial interests.

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Breast Cancer: Basic and Clinical Research

ORIGINAL RESEARCH

Delivery of MicroRNA-10b with Polylysine Nanoparticles for Inhibition of Breast Cancer Cell Wound Healing

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Abstract: Recent studies revealed that micro RNA-10b (mir-10b) is highly expressed in metastatic breast cancer cells and positively regulates breast cancer cell migration and invasion through inhibition of HOXD10 target synthesis. In this study we designed anti-mir-10b molecules and combined them with poly L-lysine (PLL) to test the delivery effectiveness. An RNA molecule sequence exactly matching the mature mir-10b minor antisense showed strong inhibition when mixed with PLL in a wound-healing assay with human breast cell line MDA-MB-231. The resulting PLL-RNA nanoparticles delivered the anti-microRNA molecules into cytoplasm of breast cancer cells in a concentration-dependent manner that displayed sustainable effectiveness.

Keywords: microRNA-10b, breast cancer metastasis, nanoparticles

Current Protocol in Toxiology

High-Throughput, Multiplexed Analysis of 3-Nitrotyrosine in Individual Proteins

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ABSTRACT

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are derived as a result of inflammation and oxidative stress and can result in protein modifications. As such, these protein modifications are used as biomarkers for inflammation and oxidative stress. In addition, modifications in single-tissue-associated proteins released into blood can provide insight into the tissue localization of the inflammation or oxidative stress. We have developed an enzyme-linked immunosorbent assay antibody microarray platform to analyze the levels of 3-nitrotyrosine in specific proteins in a variety of biological samples, including human plasma and sputum. Selective-capture antibodies are used to immunoprecipitate individual proteins from samples onto isolated spots on the microarray chips. Then, a monoclonal antibody for 3-nitrotyrosine is used to detect the amount of 3-nitrotyrosine on each spot. Our studies suggest that this approach can be used to detect trace amounts of 3-nitrotyrosine in human plasma and sputum. In this paper, we describe our antibody microarray protocol for detecting 3-nitrotyrosine in specific proteins.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION FOR LETTERS PATENT

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Compositions, Antibodies, Asthma Diagnosis Methods, and Methods for Preparing Antibodies

* * * * *

INVENTORS

Hongjun Jin
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ATTORNEY'S DOCKET NO. 16861-E-US

Compositions, Antibodies, Asthma Diagnosis Methods, and Methods for Preparing Antibodies

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/394,640 which was filed on October 19, 2010, and U.S. Provisional Application Serial No. 61/480,154 which was filed on April 28, 2011, the entirety of each of which are incorporated by reference herein.

GOVERNMENT RIGHTS STATEMENT

[0002] This invention was made with Government support under Contract DE-AC0576RLO1830 awarded by the U.S. Department of Energy. The Government has certain rights in the invention. This research was also supported by the NIEHS Exposure Biology Program (U54/ES016015) and the US Department of Defense breast cancer postdoctoral fellowship W81XWH-10-1-0031.

TECHNICAL FIELD

[0003] The present disclosure relates to the preparation of antibodies and the use thereof. Particular embodiments of the disclosure related to the preparation of antibodies having an affinity for monohalotyrosine and proteins having monohalotyrosine moieties.